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(54) Title: THE ISOLATION AND CULTURE OF FETAL CELLS FROM PERIPHERAL MATERNAL BLOOD

(57) Abstract

The present invention permits the isolation of fetal cells and in particular erythroid fetal cells from a sample of peripheral maternal blood from pregnant women, and their subsequent culture. To achieve the isolation of the above cells the sample of peripheral blood from pregnant women must be pre-enriched in nucleated cells. The pre-enriched sample of the above cells is treated with a molecule (erithropoietin) in order to establish a bond between it and The Epo-r (the specific receptor of the erithropoietin) of the fetal cells, including the erythroblasts. This molecule which has been biotinlylated at sialyl moieties permits the retrieval of fetal cells through systems of the MACS type. In fact, in such an event, magnetic particles conjugated with streptaviding join with the biotin of the erythropoietin and permit the retrieval. The cells isolated in this way can be cultivated *in vitro* for the development of CFU-E, BFU-E, GEMM-CFU cell colonies. The use of these permits prenatal molecular and cytogenetic investigation without having recourse to amniocentesis or chorionic villus sampling.

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THE ISOLATION AND CULTURE OF FETAL CELLS FROM PERIPHERAL MATERNAL BLOOD.

INTRODUCTION AND HISTORY OF THE INVENTION

A significant number of research studies have clearly demonstrated that fetal cells of various types cross the placenta and circulate in the maternal blood during pregnancy without undergoing changes from the immune system.

The development of molecular technology (PCR, FISH) and the introduction of new instruments (MACS and FACS) for the sorting of these rare fetal cells now offer a concrete possibility for their use in non-invasive prenatal genetic diagnosis.

The nucleated fetal cells of the erythroid line seem to be the most promising candidates for the achievement of this objective for a number of reasons: 1) the nucleated erythroid cells are extremely rare in adult blood and abundant in the developing fetus amounting to about 15% of the total erythrocytic mass at the ninth week of gestation and 0.5% at the nineteenth week of gestation; 2) the lifespan, at least for the most differentiated elements, would seem to be brief (approximately ninety days) which would make the survival of nucleated cells of the red series of a previous pregnancy improbable; 3) these cells are easily identifiable from the mass of maternal cells through the monoclonal antibodies for the fetal haemoglobin (anti- Y - globin).

As a consequence of the extremely diluted nature of the FNRCs (Fetal Nucleated Red Cells) in the maternal blood (one fetal cell per million maternal cells) any attempt to analyse these cells implies the need to for a previous selection by monoclonal antibodies, directed at selected surface antigens.

Combined sortings (positive and negative) have been carried out by various authors to predict the gender of the fetus, and for the retrospective diagnosis of certain chromosome pathologies (trisomy twenty-one,

eighteen, triploidy etc.) by FISH.

Depending on the type of system of cell sorting adopted and on the antigen selected, a different sensitivity has been recorded.

However in general, the purity found has been very low and the absolute number of fetal cells varies according to the antigen used. (CD 71, GPA, CD 36- Bianchi D.W.,Zickwolf G.K.,Yick.M.C.,Flint, Geifman A.F.,Ericson M.S., Williams J.M., [1993].Erythroid specific antibodies enhance the detection of fetal nucleated erythrocytes in maternal blood.Pren.Diagn.13; 293-300).

On the basis of this knowledge and state of the art we have concentrated on a pure antigen of the erythroid line, that has been able to produce more encouraging results and a higher level of purity.

This antigen has been identified as erythropoietin receptor.

Erythropoietin has been used up to the present, as a growth factor (for maturation) for nucleated cells of the red series *in vitro* and *in vivo*.

The present invention, on the other hand, utilises the antigen erythropoietin receptor for the specific purpose of carrying out the sorting of fetal cells from the sample of peripheral maternal blood taken from pregnant women and the subsequent proliferaton *in vitro* through cell cultures with CFU-E and BFU-E. clones.

DESCRIPTION

The invention in question concerns the isolation and culture of fetal cells from a sample of peripheral blood from pregnant women.

The invention involves the isolation of cells from a sample of maternal blood from pregnant women by the use of erythropoietin, a ligand molecule.

Erythropoietin binds specifically with the epo-r receptor of the erythroid fetal line thus allowing the isolation of the fetal cells from the maternal blood.

The cells isolated in this way can be cutured in vitro for the development of CFU-E, BFU-E, and GEMM-CFU cell colonies.

Therefore, recapitulating, the invention entails the use of a sample of

peripheral blood (uniparious and multiparious) from pregnant women, and the use of erythropoietin for the isolation of the fetal cells that are then used to develop fetal cell clones.

By fetal cell clones we mean the formation of cell colonies deriving from mitotic divisions of one or more cells.

By a sample of peripheral blood we mean both samples of blood from the veins and samples of blood from the arteries.

The method adopted entails the taking of a sample of peripheral blood from pregnant women, preferably of between 10 and 20 ml., and placing it in a vacutainer (BECTON-DICKINSON) which contains an anti-coagulant, preferably sodium-heparin.

The sample must be taken between the eighth and the nineteenth week of gestation, considering that at the the eighth week the fetal erythroblasts amount to 15% of the total erythrocytic mass, while at the nineteenth week they go down to 0.5%.

Before beginning the treatment with erythropoietin, the sample of peripheral maternal blood has to be pre-enriched.

A blood sample contains many types of blood cells, such as, for instance, lymphocytes monocytes, granulocyte, and non nucleated fetal cells (erythrocytes).

The non nucleated blood cells make up the majority of the cells present in the sample of peripheral blood, and it is preferable to remove these before treating the sample of peripheral maternal blood with erythropoietin.

Hence by pre-enriched we mean the formation of a sample of peripheral blood that has been enriched with nucleated cells.

It is evident that a blood sample pre-enriched with nucleated cells contains a higher percentage of nucleated cells compared with a sample of peripheral maternal blood in which the process of pre-enrichment has not been carried out.

To carry out the pre-enrichment it is necessary therefore, to separate the nucleated cells from the non-nucleated ones., and to this end the technique of gradient density centrifugation is used.

In this technique, a sample of peripheral maternal blood of pregnant women is placed in a tube containing a substance of a specific density, and the separation of the various types of cells is carried out when it is subjected to centrifugation.

There are various commercially available materials for carrying out this separation, such as, for example FICOLL (PHARMACIA, UPPSALA, SWEEN), HISTOPAQUE (SIGMA DIAGNOSTICS, ST LOUIS, MO.), Etc.

It is possible to carry out a pre-enrichment of nucleated cells also by using a solution capable of achieving the lysis of the non-nucleated cells.

Once the sample of peripheral maternal blood has been taken from pregnant women, it is diluted with PBS (calcium and magnesium free) in a 1:1 ratio; it is then stratified on Histopaque1077 (SIGMA DIAGNOSTICS, ST LOUIS, MO. USA, 10771) and is centrifuged at 400 g for thirty minutes at room temperature.

After the centrifugation, the sample of maternal blood is separated into three layers: the top layer containing serum and platelets; a layer of mononucleated cells and the layer containing erythrocytes (non-nucleated blood cells of the red series).

The layer of mononucleated cells is retrieved using, for example a Pasteur pipette.

These cells are then resuspended in PBS (phosphate buffer) and centrifuged at 200 g for ten minutes at room temperature.

After this the Pellet containing the mononucleated cells is retrieved .

These cells are then resuspended in PBS and incubated with biotinylated erythropoietin (20 nmol 1/1 for eight hours at four degrees centigrade.

The biotilynation of the erythropoietin is obtained with the following process.

The biotilynation of recombinant human erythropoietin (100.00 units/mg-SIGMA E9761) at sialyl moieties does not influence the biological properties of the molecule, or the capacity to bind with its receptor (cf. Wojchwoski D.M. and Caslake L., Blood, vol 74, n.3, 1989, pp. 952-958.)

The erythropoietin is incubated at 0 degrees centigrade in 12.5 nmol per litre of sodium metaperiodate (SIGMA-CHEMICAL CO., St Louis, Mo USA) with 0.1 nmol/L of NaCL (SERVA-30183) and 0.1 nmol/L of sodium acetate (SIGMA-CHEMICAL CO. S8750).

This process of oxydisation of the erythropoietin is carried out at ph 5.8 for ten minutes.

The erythropoietin oxidised in this way, is dialysed against eighty nmol/L of Na2HPO4 (SIGMA-CHEMICAL CO.) and 20nmol/L of NaH2PO4 (SIGMA CHEMICAL CO.) at ph 7.5 and at 0 degrees centigrade for about twelve hours in order to re-equilibrate the ph.

Subsequently the oxidised and dialysed erythropoietin is incubated for 120 minutes in agitation at 37 degrees centigrade with Biotinamyde-Caproyl-hydrazide 10 mg/ml (SIGMA-CHEMICAL CO. - St Louis ,USA) and with 20nmol/L of Na CNHB3 (SIGMA ACHEMICAL CO.-S8628).

At this point, the biotinylated erythropoietin is dialysed against PBS 0.02% - Tween-20 (SIGMA CHEMICAL CO. P-1379) at four degrees centigrade for about twelve hours, in order to eliminate the excess unbound biotyn.

The biotinylated erythropoietin produced in this way can be kept at 4 degrees centigrade for one month.

By erythropoietin we mean the protein as such, that is capable of binding with the eporeceptor.

The erythropoietin can be prepared either by purifying biological fluids (see urine) or as a biotechnological product (recombinant DNA).

In our case we also utilised E9757 (fom human urine 80.000 U/mg) and E2639 (from human urine 500 U/mg).

It is preferable to use human erythropoietin, but erythropoietin from other species have also responded in a satifactory way and could be used for the isolation of the fetal cells from maternal blood.

Recapitulating, once the Pellet containing the mononucleated cells has been retrieved, these cells are resuspended in PBS and incubated with biotinylated erythropoietin (20 nmol/L), prepared according to the process described above, for eight hours at four degrees centigrade (the condition

for equilibrium binding).

In this way the fetal cells present in the sample of mononucleated cells will bind to the biotinylated erythropoietin.

In order to obtain the highest number of cells binding to the erythropoietin (which has been biotinylated at sialyl residues) the incubation time has to be eight hours (the condition for equilibrium binding).

Presumably, in the comparative process the incubation time of one hour for the eporeceptor-erythropoietin interaction determines a substantial reduction in the binding potential (holding back) and thus reduces sensitivity.

The results of morphological and cytochemical studies conducted on samples of peripheral maternal blood of pregnant women, selected with erythropoietin eporeceptor, have recorded a homogeneous population of of erythroid elements of high purity.

In particular, the homogeneity of the population of erythroid cells can be seen using an antihaemoglobin fetal antibody (MoAb chain Hb RPCR 8115M, Europa Research Products)

It is as well to specify that by erythroid cells we mean all the cells that define the fetal erythroid line and which thus possess an eporeceptor.

Eporeceptors are in fact confined to the erythroid line and are present on the surface of the progenitor cells of the erythroid line, beginning from the BFU-E, peaking at the CFU-E and disappearing at the reticolocyte stage of maturation.

Scotchard analysis has shown the presence of 200 high affinity, and 800 of low affinity receptors in human cells at the CFU-E stage, levels which testify to the intimate interaction between the receptor and its ligand.

Once the binding of the fetal cells (present in in the sample of mononucleated cells) and the biolitynated erythropoietin has been thus carried out, these are retrieved and washed twice in a solution of PBS/BSA (0.5% bovine serum albumin), in order to remove the unbound biotinylated erythropoietin.

Subsequently the cells retrieved and washed in this way are resuspended

at a concentration of 10^7cells to 60 microlitres of buffer (PBS/BSA 0.5%) and incubated with 4O microlitres of magnetic particles conjugated with streptavidin (Myltenyl-Biotec-GMbH-Germany), for 15 minutes at 4 degrees centigrade.

The cells marked in this way are resuspended in 500 microlitres of PBS/BSA and separated from the remaining mononucleated cells by miniMACS, using a M-S type column with a G-26 flow regulator, as recommended by the manufacturers (Miltenyl-Biotec-GMbH-Germany) .

The G-26 flow regulator is placed under the miniMACS separation unit.

The column is washed by allowing 500 microlitres of PBS/BSA to flow through it and discarding the effluent.

The cells, marked and resuspended in 500 microlitres of PBS/BSA (PBS/BSA 0.5%) are then applied to the top of the column of the miniMACS, and the negative fraction is eluted with buffer (PBA/BSA 0.5%).

The column is washed with a further 500 microlitres of buffer (PBS/BSA 0.5%).

The flow speed is set at about 200 microlitres per minute.

The flow regulator is then removed and the column is washed twice with 500 microlitres of PBS/PBA (PBS/PBA 0.5%) in order to assist the eluition of the negative fraction.

In order to increase the sensitivity of retrieval of the cells marked positive, the negative fraction and the washing buffer are passed over the column a second time.

The column is then transferred onto a test tube to retrieve the positive fraction (the fetal cells).

To this end, one ml of buffer (PBS/BSA 0.5%) is placed in the top part of the column.

By using the piston supplied by the manufacturer with the miniMACS (Miltenyl-Biotec-GMbH-Germany), the fetal cells are made to flow, and then retrieved in a small volume of PBS. At this point the cells can be used for the PCR (example no.1) or cultured (examples 2 & 3).

In the second case, the separated and resuspended cells in a small volume of PBS, are placed in 35mm Petri capsules with 3 ml of Iscove's Medium containing 30% of bovine fetal serum, 1% of bovine albumin serum,10-4 M of 2- Mercaptoethanol, 0.9 % of Methylcellulose, 50 n/gml of stem cell growth factor from human recombinant DNA, 10 ng/ml of growth factor of the monocytic and granulocytic colonies (Human Recombinant GM-CSF), IL3, and three units/ml of erythropoietin; all this is placed in the thermostat at 37 degrees centigrade with 5% of carbon dioxide.

The colonies of cells are identified and counted after six days (CFU-E) and after 8-10 days (BFU-E).

They proliferate rapidly *in vitro,*and yield clearly identifiable colonies of between ne hundred and one thousand cells when cultured in a semi-solid medium together with the combinations of the cytokines specified.

In particular, in cases of aneuploidy there is a greater development of GEMM-CFU, compared with CFU-E and BFU-E.

The colonies are identified through an inverted microscope, and are harvested from the culture medium with a micro-pipette.

Each colony is dispersed in 50 microlitres of sterile PBS and centrifuged

After two to three washings with PBS (and the accompaying centrifugations), carried out to take out the residual metilcellulose, the cells are resuspended in a small volume of PBS.

At this point, that is, after the isolation and placing in culture of the fetal cells, a high number of cells is available, sufficient to permit the most varied microscopic investigations of traditional diagnostic practice.

Through the PCR, The FISH and the metaphase plates of the erythroid cells isolated it is possible to test and register the efficacy of the isolation.

The present invention is accompanied by the following examples, which do not, however constitute a limitation of application, but are intended to describe its efficacy, and the results obtained.

EXAMPLE 1

The use of PCR (Polymerase Chain Reaction) on the sample of isolated fetal cells.

The present invention allows the isolation of a high number of fetal cells from a sample of peripheral maternal blood taken from pregnant women, through the use of erythropoietin.

In this example we use a sample of maternal pregnant women with male fetuses, whose sex had been predicted by ecography.

The confirmation that the isolation of the fetal cells had taken place from the maternal ones was recognized through the amplification of segments of DNA in locus of a Y chromosome.

The PCR was carried out using Primers WYR007 and WYR008 which amplify a DNA segment by 124 bp of the repetitive sequence DYZI locus of the Y chromosome.

All the reactions were carried out in sterile conditions.

Any possible contaminations of external male DNA were always tested for through negative tests of female DNA from non pregnant women and tests of the reagents,with no DNA added, for each reaction.

In order to increase sensitivity and specificity of PCR amplification,an anti-Taq Start antibody was utilised in each reaction,with the concentrations and procedure recommended by the manufacturer (Clontech Laboratories Inc,Palo Alto, USA)

Taq start antibody is used to block polymerase activity during the set up of PCRs at room temperature (20-22 degrees centigrade), thus preventing the proliferation of amplification.

The inhibition of Taq polymerase is completely anulled when the temperature is raised above 70 degrees centigrade.

The cells isolated by the use of erythropoietin and resuspended in PBS, can be used for PCR right away, or they can be frozen at 20 degrees centigrade for subsequent investigation.

In this last case, the cells have to be thawed at 37 degrees centigrade. Then they are boiled for five minutes to extract the DNA.

The PCR is carried out in a volume of 2.5 microlitres of Buffer 1OX (Cetus Perckin Elmer- Roche Molecular System USA), one microlitre of nucleotides (0.1 mM final concentration), 2 microlitres of magnesium

chloride (2 mM final concentration), 2 microlitres of each primer (0.25 microlitres per Primer), and 0.2 units of Taq polymerase.

Sixteen microlitres of the denaturated cellular lysate is added to the main solution and the amplification is carried out for 32 cycles at 94 degrees centigrade for one minute, at 56 degreees centigrade for one minute and at 72 degrees centigrade for one minute.

The first denaturation cycle was carried out at 94 degrees centigrade for six minutes and the extension time of the final cycle was of ten minutes at 72 degrees centigrade.

After the amplification, the samples were separated by electrophoresis with a a 2% Agarose gel.

The presence of 124 bp fragment is considered significant only if the female and segment tests are negative.

As a test of oligospecificity and of the cell isolation, negative sorted factions are amplified under the conditions specified.

The presence of the 124 bp fragment of the locus of the Y chromosome unequivocally indicates the presence of male fetal cells.

The following chart indicates the number of cases examined together with the exemplification described and the results obtained.

Amplification by PCR of the DYZI sequence specific of the Y chromosome from a sample of peripheral maternal blood.

Patient	Week of gestation	Blood ml	PCR* Epo-r	Sex of fetus
1	15	15	+	M
2	15	15	+	M
3	15	14	+	M
4	13	12	+	M
5	15	12	+	M
6	17	14	+	M
7	15	12	+	M
8	16	14	+	M

9	12	14	+	M
10	17	15	+	M
11	15	14	+	M
12	15	16	+	M
13	16	12	+	M
14	16	14	+	M
15	15	15	+	M
16	12	16	+	M
17	14	14	+	M
18	14	15	+	M

* Obvious presence of a 124 bp fragment.

EXAMPLE No. 2

Use of FISH (in situ hybridization) on the sample of isolated fetal cells.

In this example a sample of peripherical maternal blood of a pregnant woman was utilised in which, through the use of traditional methods, the presence of a Down's Syndrome fetus (trisomy 21) had been identified.

For the study of the trisomy 21 a commercially available biotinylated cosmid probe specific for the region of Down's Syndrome 21 q22 (locus D21 S65) was used. Beginning with the cell colonies isolated according to the present invention, the coverslips were prepared by cytocentrifugation.

These were then fixed in glacial acetic methanol-acid (3:1) for thirty minutes at room temperature, without the preceding treatment with hypotonic solution.

The hybridization was carried out according to the manufacturer's instructions (CYTOCELL, UK).

Fluorescinated avidin was used for the identification of the signal, which was amplified by using biotinylated goat antiavidin, with a final layer of fluorescinated avidin.

For both probes the slides were mounted in in a 90% solution of glycerol containing 2,33 % of DABCO [1.4 diazabilciclo-2.2.2] ottano: Sigma Chemical Co.) as antifade medium; with 0.1 microgrammes per ml Propidium Iodide for counterstaining.

The slides produced in this way were examined under an epifluorescent microscope eqiupped with a CCD camera.

A minimum of 200 nuclei were counted for each case analysed.

In about 80% of the nuclei analysed the three clear signals of trisomy were evident.

Overlapping nuclei were not considered.

Thus, from a sample of peripheral maternal blood from a woman pregnant with a Down's Syndrome fetus, through the procedure described, it was possible to show the presence of trisomy 21 in the isolated fetal cells; this fully confirms the efficacy of the method of isolation, which is the subject of the present invention.

Through the example above, the efficacy of isolation of the fetal cells from the sample of the maternal blood has been verified, with resulting levels of purity of up to 80% of fetal cells, as shown in the following chart.

Chart 2

Data from two samples of peripheral maternal bloodfrom women pregnant with a trisomy 21 fetus (47 XY+21), treated with erythropoietin and studied by PCR/FISH and fetal haemoglobin.

Case 1

Stage of gestation	Nineteenth week
Volume of blood	15ml
No. of days of culture	11
Types of colony	(> l00) CFU-GEMM
No. of cells	3×10^5
HbF+ (%)	Not Done
FISH 21 signal (%)	
0	-
1	-
2	16
3	70
4	14

Sex for PCR*	+

Case 2

Stage of gestation	twenty-fifth week
Volume of blood	12ml
No. days of culture	7
Types of colonies	CFU-GEMM(80)/CFU-E (20)
No. of cells	2×10^5
No, of HbF+ (%)	25%
FISH 21 signal %	
0	-
1	-
2	60
3	40
4	-
Sex for PCR*	+

* Presence of a clear fragment of124 bp.

EXAMPLE no.3

The preparation of metaphasic plates from fetal cells isolated with erythropoietin and cultured in vitro.

CFU-E colonies can be treated after about six days and indeed this is the best time to obtain the best mitotic harvest.

BFU-E colonies for the same reason are treatable after about 8 days.

Thus, after a period of between six and ten days of cell culture, to the Petri capsule containing the fetal cells in the culture medium are added 50 microlitres of COLCEMID (Karyiomax Colcemid Solution, 10 microgrammes/ ml, GIBC0 BRL) for about 8 to 12 hours.

After this time, the cells are retrieved and washed two or three times in PBS, to remove the residual metylcellulose and the remaining components of culture medium.

Then the cells in PBS are centrifuged for ten minutes at 1000 RPM and retrieved by discarding the top layer and taking out the pellet.

The pellet containing the fetal cells is placed in a 15ml tube with 0.075 M of KCL hypotonic solution, and is agitated very delicately with a pipette and placed in a water bath at 37 degrees centigrade for about ten minutes.

After this period the tube containing the cells is centrifuged for about ten minutes at 1000 RPM.

The top layer is discarded, and the pellet is treated with a solution of cold methanol and glacial acetic acid in a 3 to 1 ratio. (cell fixing)

After about 60 minutes this is recentrifuged and another treatment with 3 to1 of methanol-glacial acetic acid is given.

This last treatment is repeated another three times.

After the final centrifugation, the pellet containing the cells is resuspended in a small volume of fixative (methanol-glacial acetic acid 3:1) and removed with a Pasteur pipette.

With the same pipette, which contains the cells, the slides are prepared by the drop on drop system, allowing them to fall on the glass.

This system favours the spreading (opening) of the metaphasic plates (mitosis).

The slides prepared in this way, are air dried for about ten minutes and then examined with an inverted microscope to verify the presence of the metaphasic plates of the fetal cells.

After this examination the slides can be coloured GIEMSA MAYGRUNWALD (coloured metaphasic plates), or banded and subsequently coloured (banded metaphasic plates).

At this point the slides are ready for observation through a microscope.

CLAIMS

1. A method for the isolation and retrieval of fetal cells from a sample of peripheral maternal blood of pregnant women which includes:

-a sample of peripheral maternal blood from pregnant women;

-the pre-enrichment of the peripheral maternal blood sample of pregnant women in nucleated cells;

- the isolation of the fetal cells from the peripheral blood sample from pregnant women, which has been previously pre-enriched by a molecular ligand;

- the culture of fetal cells through the use of a cultural medium .

2. The method referred to in the above claim No.1, wherein is carried out the isolation of fetal cells, e.g fetal erythroblasts, from the sample of uniparious and multiparious peripheral maternal blood of pregnant women.

3. A method of pre-enrichment of nucleated cells as distinct from non-nucleated cells, in a sample of peripheral blood from pregnant women.

4. The method referred to in preceding claim No.3, wherein this is carried out through the separation of nucleated cells from nonnucleated cells.

5. The method referred to in preceding claim No. 4 wherein the separation of nucleated cells from non-nucleated cells is carried out by gradient density centrifugation.

6. The method referred to in preceding claim No. 5 wherein for the gradient density centrifugation Ficoll is used .

7. The method referred to in preceding claim No. 5 wherein for the centrifugation of gradient density centrifugation Histopaque is used.

8. The method referred to in preceding claim No.5 wherein for the gradient density centrifugation Polymorphprep is used.

9. The method referred to in preceding claim No.5 wherein for the gradient density centrifugation Nicodenz is used.

10. The method referred to in preceding claim No.4 wherein the separation of nucleated cells from non-nucleated cells is carried out through solutions for the lysis of the non-nucleated cells (erythrocytes).

11. A method for the pre-enrichment of the sample of peripheral maternal

blood from pregnant women in erythroid fetal cells as distinct from the non-nucleated cells, by using gradient density centrifugation.

12. The method referred to in the preceding claim No.11 wherein it is carried out by the separation of the erythroid cells from the non-nucleated cells.

13. The method referred to in the preceding claim No.12 wherein the separation of the erythroid cells from the non-nucleated cells is carried out by gradient density centrifugation .

14. The method referrred to in preceding claim No. 13 wherein for the gradient density centrifugation, Ficoll is used.

15. The method referred to in the preceding claim No.13 wherein for the gradient density centrifugation, Histopaque is used.

16. The method referred to in the preceding claim No.13 wherein for the gradient density centrifugation, Polymorphprep is used.

17. The method referred to in the preceding claim No.13 wherein for the gradient density centrifugation Nycodenz is used.

18. The method referred to in the preceding claim No.11 wherein the separation of fetal erythroid cells from non-nucleated cells is carried out through the use of solutions for the lysis of the non-nucleated cells. (erythrocytes).

19. A method for the isolation of fetal cells from a sample of peripheral maternal cells of pregnant women through the use of erythropoietin which includes:

- a sample of peripheral maternal blood from pregnant women;
- the pre-enrichment of the sample peripheral maternal blood from pregnant women by the use of a gradient density centrifugation;
-the isolation of fetal cells from the pre-enriched sample of peripheral blood from pregnant women through the use of erythropoietin;
-the culture of the isolated fetal cells through~he use of a culture medium.

20. The method referred to in preceding claim No. 19 wherein human erythropoietin is used for the isolation of the fetal cells.

21. The method referred to in preceding claim No. 20 wherein biotinylated

human erythropoietin is used for the isolation of the fetal cells.

22. The method referred to in preceding claim No. 21 wherein human biotinin-(sialyl) erythropoietin is used for the isolation of the fetal cells.

23. The method referred to in preceding claim No. 22 wherein human erythropoietin conjoined to the Epo-r receptor (the specific receptor for erythropoietin) is used for the isolation of the fetal cells.

24. The method referred to in preceding claim No. 23 wherein biotinylated erythropoietin is used for the isolation of the fetal cells.

25. The method referred to in preceding claim No. 19 wherein erythropoietin different from human erythropoietin is used for the isolation of the fetal cells.

26. The method referred to in preceding claim No.25 wherein non-human biotinylated erythropoietin is used for the isolation of the fetal cells.

27. The method referred to in preceding claim No.26 wherein non-human biotin (-sialyl) erythropoietin is used for the isolation of the fetal cells.

28. The method referred to in preceding claim No.27 wherein non-human erythropoietin, conjoined to the receptor Epo-r (the receptor specific for erythopoietin) is used for the isolation of the fetal cells.

29. The method referred to in preceding claim No.28 wherein non-human biotinylated erythropoietin is used for the isolation of the fetal cells.

30. A method for the isolation of the fetal erythroblasts from a sample of peripheral maternal blood from pregnant women through the use of erythropoietin, which includes:

- a sample of peripheral blood from pregnant women;
- the pre-enrichment (in non-nucleated cells) of the sample of peripheral maternal blood taken from pregnant women;
-the isolation of the fetal erythroblasts from the sample of peripheral blood taken from pregnant women by the use of erythropoietin;
- the culture of the isolated fetal erythroblasts. through the use of a cultural medium.

31. The method referred to in the preceding claim No. 30 wherein for the isolation of the fetal erythroblasts human erythropoietin is used.

32. The method referred to in the preceding claim No. 31 wherein for the isolation of the fetal erythroblasts, human bitinylated erythropoietin is used.

33.The method referred to in the preceding claim No. 32 wherein for the isolation of the fetal erythroblasts, human biotin (- sialyl) erythropoietin is used.

34.The method referred to in the preceding claim No. 33 wherein for the isolation of the fetal erythroblasts, human erythropoietin conjoined to the receptor Epo-r (the specific receptor for erythropoietin) is used.

35.The method referred to in the preceding claim No. 34 wherein for the isolation of the fetal erythroblasts, biotinylated erythropoietin is used.

36.The method referred to in the preceding claim No. 30 wherein for the isolation of the fetal erythroblasts, non-human erythropoietin is used.

37.The method referred to in the preceding claim No. 36 wherein for the isolation of the fetal erythroblasts, non-human biotinylated erythropoietin is used.

38.The method referred to in the preceding claim No. 37 wherein for the isolation of the fetal erythroblasts, non-human biotin (- sialyl) erythropoietin is used.

39.The method referred to in the preceding claim No. 38 wherein for the isolation of the fetal erythroblasts, non-human erythropoietin conjoined to the receptor Epo-r (the specific receptor for erythropoietin) is used.

40.The method referred to in the preceding claim No. 39 wherein for the isolation of the fetal erythroblasts, non-human biotinylated erythropoietin is used.

41. The use of the conjunction of biotin-streptavidin for the isolation of fetal cells from a sample of peripheral maternal blood from pregnant women.

42. The use of the conjunction referred to in preceding claim No. 41, wherein the same is used to isolate fetal erythroblasts.

43. A method for the isolation of fetal cells from a sample of peripheral maternal blood from pregnant women by the use of streptavidin conjoined

to human biotinylated erythropoietin.

44. The method referred to in preceding claim No. 43 wherein the erythropoietin is of non-human type.

45. A method for the isolation of fetal erythroblasts from a sample of peripheral maternal blood from pregnant women through the use of streptavidin conjoined to human biotiylated erythropoietin .

46. The method referred to in preceding claim No. 45 wherein the erythropoietin used is non-human.

47. A method for the isolation of fetal cells from a sample of peripheral maternal blood from pregnant women by the use of magnetic particles.

48. The method referred to in the preceding claim No. 47 wherein, by the isolation of the fetal cells the magnetic particles conjoin with the streptavidin.

49. The method referred to in preceding claim No. 48 wherein, through the isolation of the fetal cells, the magnetic particles already conjoined to the streptavidin conjoin with the biotin conjoined with the sialyl residues of the erythropoietin.

50. The method referred to in the preceding claim No. 49 wherein, through the isolation of the fetal cells, the magnetic particles conjoined with the streptavidin and with the biotin conjoined with the sialyl residues of the erythropoietin, through this last, conjoin with the fetal cell.

51. A method for the isolation of the fetal erythroblasts from a sample of of peripheral maternal blood from pregnant women through the use of magnetic particles.

52. The method referred to in preceding claim No. 51 wherein for the isolation of the fetal erythroblasts the magnetic particles conjoin with streptavidin.

53.The method referred to in preceding claim No. 52 wherein for the isolation of the fetal erythroblasts the magnetic particles already conjoined with the streptavidin, through this last, conjoin with the biotin that is conjoined with the sialyl residues of the erythropoietin.

54.The method referred to in preceding claim No. 53 wherein for the

isolation of the fetal erythroblasts the magnetic particles conjoined with the streptavidin and the biotin conjoined with the sialylresidues of the erythropoietin, through this last, conjoin with the fetal cells.

55. The culture of the fetal cells isolated through the use of erythropoietin from a sample of peripheral maternal blood of pregnant women.

56.The culture of the fetal erythroblasts isolated through the use of erythropoietin from a sample of peripheral maternal blood of pregnant women.

57. The culture of the cells referred to in preceding claim No. 55. and 56. wherein this is carried out in order to increase the number of cells isolated.

58. A method for the isolation of fetal cells in metaphase from a sample of peripheral maternal blood of pregnant women which includes:

-a sample of peripheral maternal blood of pregnant women;

- the pre-enrichment of the sample of nucleated cells;

- the treatment of the sample with erythropoietin;

- the culture of the isolated fetal cells from the sample.

- exposing the culture to an agent that inhibits the division of the cells through the interruption of the division at the metaphase stage;

-exposing the cell cultures to a growth synchronizing agent

- the harvesting of the metaphase fetal cells;

59. The method referred to in preceding claim No.58. wherein the isolated fetal cells are erythroblasts.

60. The method referred to in preceding claims Nos.58 and 59 wherein Colcemid is utilised as the agent that inhibits the continuing division of the cells in the cell cycle.

61. The method referred to in preceding claims Nos.58 and 59 wherein Colchicina is utilised as the agent that inhibits the continuing division of the cells in the cell cycle.

62. The method referred to in preceding claims Nos.58 and 59 wherein Viniblastin salts are is utilised as the agent that inhibits the continuing division of the cells in the cell cycle.

63. The method referred to in preceding claims Nos.58 and 59 wherein the

growth synchronizing agent used is bromodeoxyuridin.

64. The method referred to in preceding claims Nos.58 and 59 wherein the growth synchronizing agent used is fluorodeoxyuridin.

65. The method referred to in preceding claims Nos.58 and 59 wherein the growth synchronizing agent used is ethidium bromide.

66. The use of human GM-CSF as a growth factor in the fetal cell colonies isolated through the use of erythropoietin from a sample of peripheral maternal blood taken from pregnant women.

67. The use of human GM-CSF as a growth factor in the erythroid fetal cell colonies isolated through the use of erythropoietin from a sample of peripheral maternal blood taken from pregnant women.

68. The use of GM-CSF of non-human type for the purposes specified in preceding Claims Nos.66 and 67.

69. The combination of erythropoietin with biotin, carried out in order to isolate fetal cells from a sample of peripheral maternal blood taken from pregnant women.

70. The combination referred to in preceding claim No.69 wherein it is carried out in order to isolate fetal erythroblasts.

71. The use of biotinylated erythropoietin in order to conjoin with the epo-receptor present on the surface of the fetal cells.

72. The use of biotinylated erythropoietin in order to conjoin with the epo-receptor present on the surface of the fetal eryth- roblasts .

73. The use of biotinylated erythropoietin in order to isolate the fetal cells from a sample of peripheral maternal blood taken from pregnant women through the use of MACS type systems.

74. The use of biotinylated erythropoietin in order to isolate the fetal erythroblasts from a sample of peripheral maternal blood taken from pregnant women using MACS type systems.

75. The use of stem cell factor for the development of colonies of fetal cells isolated through the use of erythropoietin from a sample of peripheral maternal blood taken from pregnant women.

76. The use of stem cell factor for the development of colonies of erythroid

cells isolated through the use of erythropoietin from a sample of peripheral maternal blood taken from pregnant women.

77. The use of Interleukyn 3 for the development of fetal cell colonies isolated through the use of erythropoietin from a sample of peripheral maternal blood taken from pregnant women.

78.The use of Interleukyn 3 for the development of erythroid fetal cell colonies isolated through the use of erythropoietin from a sample of peripheral maternal blood taken from pregnant women.

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/IT 97/00162

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 16452 A (CELLPRO INCORPORATED) 31 October 1991 see the whole document ---	1-78
X	WO 96 09409 A (MILTENYI BIOTECH, INC.) 28 March 1996 see the whole document ---	1-18
X	WO 95 26417 A (GENZYME CORPORATION) 5 October 1995 see the whole document ---	1
X	WO 95 08646 A (NEW ENGLAND MEDICAL CENTER HOSPITALS) 30 March 1995 see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 November 1997	04/12/1997

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Interr nal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	BIANCHI D.W. ET AL.: "Erythroid-specific antibodies enhance detection of fetal nucleated erythrocytes in maternal blood" PRENATAL DIAGNOSIS, vol. 13, no. 4, April 1993, pages 293-300, XP002047417 cited in the application	1-78
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